

IgE enhances FcεRI expression and IgE-dependent TNF-α release from canine skin mast cells

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Abstract

The role of IgE on mast cell (MC) activation is well known. Recent studies have demonstrated that IgE also has the ability to up-regulate the high affinity IgE receptor (FcεRI) on the surface of human and murine MC, leading to an increased production of cytokines and chemokines. In the present study, we have examined the influence of IgE levels on FcεRI expression, and its consequences on TNF-α production from canine skin MC.

Mature MC were enzymatically dispersed from the skin biopsies of 6–8 dogs and were cultured for up to 5 days in medium supplemented with recombinant canine stem cell factor (SCF) (6 ng/ml), in the presence of increasing serum IgE concentrations (ranging from 0 to 80 µg/ml). Subsequently, skin MC were activated with anti-IgE, and TNF-α concentration was assessed 5 h post-activation by a cytotoxic bioassay. FcεRI receptors were identified in MC surface by flow cytometry.

MC cultured for up to 5 days in the presence of high serum IgE concentration (8 µg/ml) produced twice the quantity of TNF-α than MC cultured in the absence of serum IgE, in response to stimulation with anti-IgE. Moreover, the percentage of FcεRI-positive skin cells was found to be approximately double in cells cultured with serum IgE compared to that cultured in the absence of IgE, following saturation of IgE receptors. These results suggest that, as found in human and murine MC, IgE may induce an up-regulation of the FcεRI density and an enhancement in the secretory activity of canine skin MC. This study could be of great interest in designing new therapeutic strategies for controlling MC activation in inflammatory and allergic processes. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The binding of IgE to the high affinity receptor (FcεRI) on mast cell (MC) and basophil surface and its subsequent crosslinking by an allergen is the first step in the cascade of reactions leading to the release of cellular pro-inflammatory mediators (Shaikh et al., 1997).

In addition to its crucial role in MC activation, IgE has been shown to regulate the FcεRI expression on the surface of basophils (Lantz et al., 1997;

Abbreviations: DMEM, Dulbecco's modified Eagle's minimum essential medium; FcεRI, high affinity IgE receptor; MEM, minimum essential medium; MC, mast cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PE, phycoerythrin; SCF, stem cell factor; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor

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MacGlashan et al., 1998) and MC (Yamaguchi et al., 1997, 1999; Welker et al., 1997a,b; Shaikh et al., 1997). The first evidence for this phenomenon was reported by Conroy et al. (1977), who found a positive correlation between the serum IgE concentration and the level of FcεRI expression on human basophils. Subsequent studies on murine MC showing a similar relationship between IgE and FcεRI density demonstrated that the IgE-dependent enhancement of MC FcεRI expression allows MC to release increased amounts of pro-inflammatory mediators (serotonin, IL-6, IL-4) in response to IgE-dependent activation (Yamaguchi et al., 1997). Recently, similar results were observed in human nasal MC (Pawankar and Ra, 1998), in the human MC line HMC-1 (Welker et al., 1997a,b) and in human bone marrow derived MC (Yamaguchi et al., 1999), where an increase in the IgE-dependent release of histamine, tryptase and lipid mediators was observed after culturing the cells with high IgE concentrations. As a consequence of these observations, and given the potential clinical implications derived from an IgE-driven upregulation of IgE receptors, novel therapeutic approaches aiming at lowering the concentration of functional IgE have recently been investigated (Fahy et al., 1997; MacGlashan et al., 1997; Saini et al., 1999).

Up to date, the influence of serum IgE concentration on the secretory function of mature canine MC has not been elucidated. We recently compared the histamine releasing ability of mature canine MC isolated from the skin of allergic dogs and control dogs (exposed to high and normal serum IgE concentration, respectively), finding no significant differences between the amount of the preformed mediator released after stimulating them through the FcεRI receptor (Brazís et al., 1998). One of the possible explanations suggested in view of the results obtained was that the intracellular mechanisms leading to the release of a preformed mediator such as histamine were probably not affected by an FcεRI upregulation. Moreover, in agreement with our data, studies performed in murine MC had previously shown that the enhanced mediator release induced by an IgE-dependent FcεRI upregulation was especially remarkable in cytokine production compared to the preformed mediator release (Yamaguchi et al., 1997). We therefore suggested that, if an IgE-induced increase of FcεRI density occurred in canine skin MC, this might have a higher impact on the release of newly formed mediators such as cytokines.

In the present work, we analyzed the influence of serum IgE concentration on canine MC FcεRI expression and its consequences on MC cytokine production. The aim of the present study was therefore to evaluate the FcεRI expression on the surface of mature MC isolated from dog skin after being cultured with increased serum IgE concentration and to analyze whether the addition of serum IgE in the culture medium of this cellular population influences the MC secretory function by analyzing their TNF-α production after immunological stimulation with anti-canine IgE.

2. Materials and methods

2.1. Reagents

Collagenase, hyaluronidase, pronase, bovine albumin, soybean trypsin inhibitor (STI), cycloheximide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), calcium ionophore A23187 and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma Chemical (Madrid, Spain). Penicillin–streptomycin, Dulbecco's modified Eagle's minimum essential medium (DMEM), RPMI medium, foetal calf serum (FCS), glutamine, hepes, trypsin–EDTA and sodium dodecyl sulfate (SDS) were obtained from Gibco (Life Technologies, Barcelona, Spain). Purified polyclonal anti-canine IgE and dog IgE VET-RID plates were purchased from Bethyl Laboratories (Montgomery, TX). Monoclonal anti-canine IgE was kindly supplied by Dr. Hammerberg (College of Veterinary Medicine, NC). PE-conjugated rabbit anti-mouse IgG antibody was obtained from DAKO (Denmark). Recombinant murine TNF-α was obtained from Genzyme (Cambridge, MA). Recombinant canine SCF was kindly provided by Dr. Morstyn (Amgen, Thousand Oaks, CA).

2.2. Skin MC isolation and culture

Mature MC were enzymatically dispersed from skin biopsies taken from the abdominal area of eight healthy mixed-breed male dogs euthanized in the Barcelona Animal Pound. Briefly, skin samples were chopped into 1 mm³ fragments, and were incubated for 180 min in 15 ml of MEM per gram of skin containing 22.3 mg collagenase, 18 mg hyaluronidase, 12 mg pronase

supplemented with bovine albumin and antibiotics. After digestion, cutaneous cells were cultured for up to 5 days in complete medium (DMEM containing 100 U/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml gentamycin, 5% FCS, 6 ng/ml recombinant canine SCF) and in the presence of different canine IgE concentrations (final concentrations ranging from 0 to 80 µg of IgE/ml medium). The IgE-rich serum used for all the experiments was obtained from a pool of serums from four Beagle dogs spontaneously sensitized against *Ascaris suum* and it was heated for 30 min at 56 °C to inactivate the complement. The canine serum IgE concentration was assessed using an immunoprecipitation test (Bethyl VET-RID plates). To specifically assess the contribution of IgE on both cytokine release and FcεRI expression, control experiments were performed in parallel, where canine serum was heat-inactivated (56 °C/4 h) in order to denature the Fc-IgE chain.

2.3. Mast cell activation

To determine whether the addition of serum IgE in the culture medium influenced the MC TNF-α production, a pilot experiment was performed, where the cells were cultured for 5 days in complete medium supplemented with different serum IgE concentrations (0, 0.8, 8 and 80 µg IgE/ml medium). Harvested cells were washed twice in PBS without Ca²⁺ and Mg²⁺, and cellular viability and MC numbers were assessed before stimulation. After the culture period with either IgE-rich serum or heat inactivated IgE serum, cells were sensitized to allow a maximal FcεRI saturation by incubation in DMEM in the presence of 5% of IgE-rich serum for 2 h at 37 °C. After sensitization, cells were washed twice again and resuspended in DMEM containing STI (200 µg/ml) in the absence of FCS. Activation was performed by incubating the cells (2 × 10⁴ MC/100 µl) with polyclonal anti-canine IgE (1 µg/ml) after a 15 min preincubation period with SCF (50 ng/ml). Cell supernatants were collected at 5 h after stimulation to assess TNF-α activity by a cytotoxicity bioassay. All experiments were performed in duplicates.

2.4. Cytotoxicity bioassay

TNF-α activity was assessed in cell supernatants using a cytotoxicity bioassay modified from a

method previously described (Gordon and Galli, 1991). Briefly, cells from the TNF-α-sensitive L929 (mouse fibroblast) cell line were seeded (5 × 10⁴ cells/well) into 96-well-plates and cultured overnight in RPMI medium supplemented with 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% hepes 1 M. The medium was then replaced with 50 µl/well of fresh medium containing 5 µg/ml of cycloheximide and 100 µg/ml of STI. Mouse recombinant TNF-α was used to construct a standard curve ranging from 2 to 2000 pg/ml. Fifty microliter per well of samples and standards were added, and plates were incubated for 18 h at 37 °C. Then 10 µl/well of MTT (5 mg/ml) was added, and, following a 4 h incubation, 50 µl of a solution of 50% N,N-dimethyl-formamide and 20% SDS, pH 7.4, was added to dissolve the MTT; the mixture was then incubated overnight. The plates were read in an ELISA reader at 570 nm.

2.5. IgE receptor expression

In order to assess the expression of FcεRI on the surface of cutaneous cells, a cytometric analysis was carried out. After a 5-day-culture period with either normal canine serum IgE (8 µg/ml), or heat-inactivated serum, aliquots of 5 × 10⁵ cells were sensitized for 1 h at 4 °C in medium containing 5% IgE-rich dog serum, in order to fully saturate any Fc-IgE receptor expressed by the cells. To check the specificity of the staining, control aliquots were incubated either with heat-inactivated IgE serum or in the absence of IgE. After washing twice, all cells were incubated for 30 min at 4 °C with 100 µl of monoclonal anti-canine IgE (10 µg/ml PBS). Duplicates were incubated under the same conditions with 100 µl of isotype control (10 µg/ml of mouse IgG). Cells were then washed again and incubated for another 30 min with 100 µl of rabbit anti-mouse IgG antibody conjugated to PE at 4 °C in the dark. After washing twice, cells were fixed with 200 µl of PBS containing 1% formaldehyde. Stained cells were analyzed using a FACSCalibur (Becton Dickinson, Spain).

2.6. Data analysis

Mediator release results are expressed as the arithmetical mean ± standard error of the mean (mean ±

SEM) of eight experiments. Differences between means were tested for significance by Student's t-test at a level of significance of 0.05.

3. Results

3.1. Dose-dependent effect of canine serum IgE concentration on IgE-dependent MC TNF- α production

Fig. 1 shows the TNF- α production of MC cultured for up to 5 days in the presence of increasing serum IgE concentration (0–80 μ g IgE/ml medium). Before activation, cells were passively sensitized for 2 h with DMEM containing 5% of IgE-rich serum. Stimulation was carried out by incubating the cells for 15 min with SCF (50 ng/ml), followed by anti-IgE (1 μ g/ml), and cell supernatants were collected at 5 h post-activation. A dose-dependent correlation between the concentration of serum IgE added to the cell culture medium and the concentration of TNF- α released by the skin MC was observed. Maximal TNF- α release (12.44 ± 1.85 pg/ml) was achieved when cells were cultured with 8 μ g/ml of serum IgE.

3.2. Effect of serum IgE concentration on IgE-dependent and IgE-independent MC TNF- α production

In order to specifically assess the contribution of IgE (added to the culture medium) to the cytokine production, we compared the effect of culturing the cells with either serum IgE (optimal concentration of 8 μ g IgE/ml medium) or heat-inactivated serum IgE on the TNF- α production after stimulation through the Fc ϵ RI (using anti-IgE) (Fig. 2a). After the culture period and before activation, all cells were sensitized for 2 h with IgE-rich serum in order to achieve a maximal saturation of IgE receptors present on the mast cell membrane. As shown in Fig. 2a, we found that cells that had been cultured for 5 days with serum IgE (8 μ g/ml) produced a significantly higher Fc ϵ RI-dependent TNF- α concentration (12.44 ± 1.85 pg/ml), in comparison with cells that had been cultured with heat-inactivated serum IgE (6.87 ± 1.87 pg/ml). The increased TNF- α production on MC cultured with IgE-rich serum did not reflect a general enhancement of the cells' secretory response, in that the TNF- α release on both cell populations (cultured with normal or heat inactivated serum IgE) activated through an Fc ϵ RI-independent secretagogue (calcium ionophore

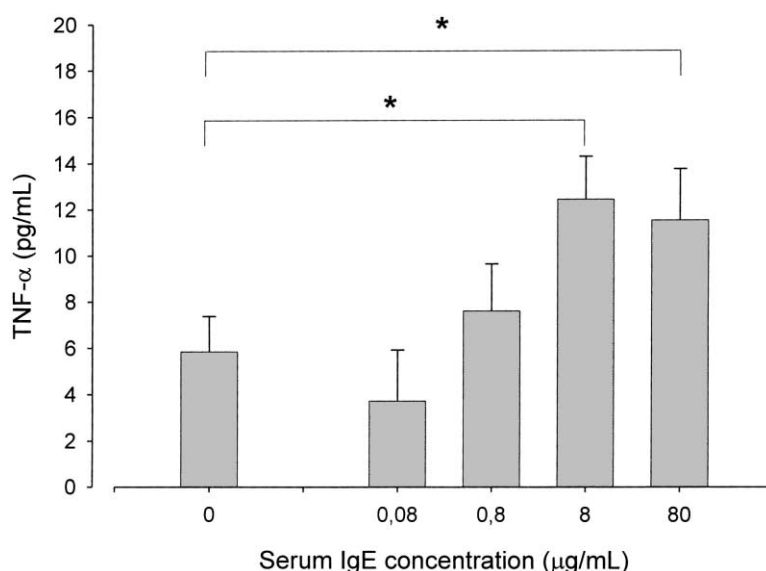


Fig. 1. Dose-dependent TNF- α release from skin MC cultured for 5 days with increasing serum IgE concentrations and activated upon anti-IgE (1 μ g/ml) (* $p < 0.05$). Each bar represents the mean $\pm$ SEM of eight independent experiments.

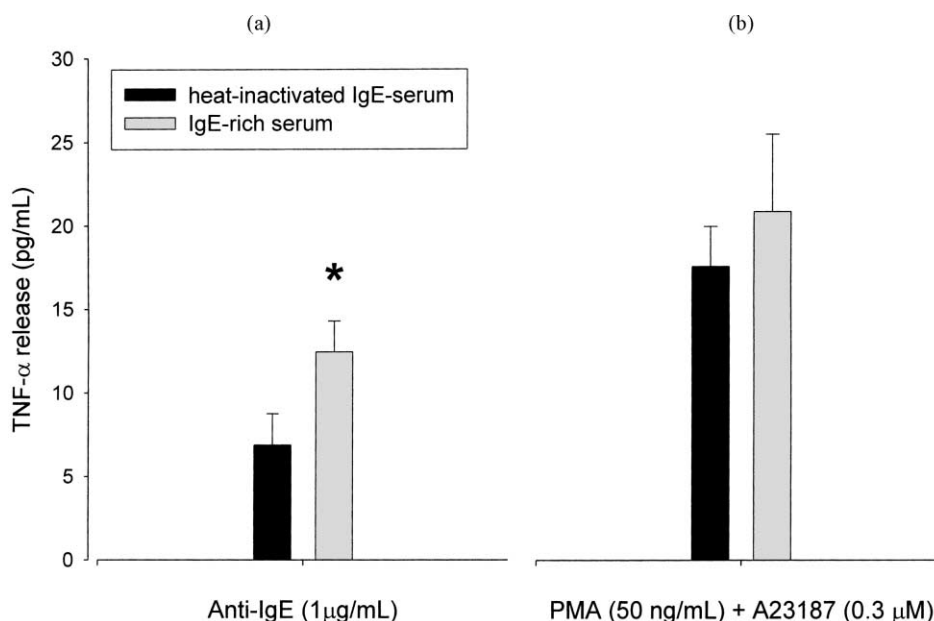


Fig. 2. (a) TNF- α release from skin MC cultured in the presence (gray bars) or in the absence (black bars) of IgE (8 μ g/ml) activated upon IgE-dependent (1 μ g/ml anti-IgE); (b) IgE-independent (50 ng/ml PMA + 0.3 μ M A23187) stimuli. Each bar represents the mean $\pm$ SEM of six independent experiments.

A23187 and PMA) was not significantly affected (Fig. 2b). The difference in the IgE-mediated TNF- α production on MC may therefore reflect the difference in the expression of the Fc ϵ RI on the cell membrane.

3.3. Fc ϵ RI expression analysis

3.3.1. Detection of Fc ϵ RI positive cells

As shown in Fig. 3a, we identified the cutaneous Fc ϵ RI positive cells through a flow cytometric analysis. In order to detect the population of dermal cells

capable of binding IgE, cells were analyzed by flow cytometry after being passively sensitized with 5% IgE-rich serum medium. To double check the specificity of the staining method, cells were sensitized in parallel with heat-inactivated serum IgE (where IgE had lost their ability to bind to its receptor) (Fig. 3b).

3.3.2. Effect of IgE serum in MC Fc ϵ RI expression

To analyze the MC Fc ϵ RI expression, cutaneous cells were cultured for up to 5 days in complete medium containing either canine IgE-rich serum

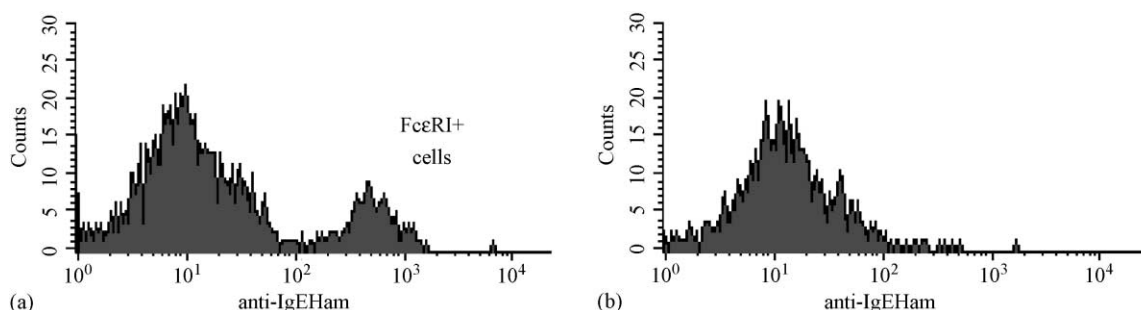


Fig. 3. Identification of skin IgE-bound cells through a flow cytometric analysis. Cells were sensitized for 1 h with (a) or without (b) IgE-rich serum. Cells were then stained with monoclonal anti-canine IgE (4 °C for 30 min) and PE-conjugated rabbit anti-mouse IgG (4 °C for 30 min).

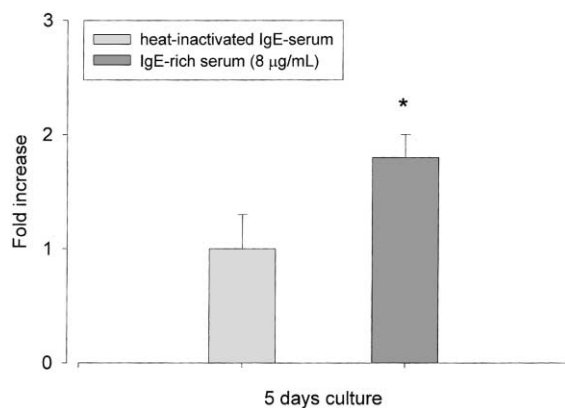


Fig. 4. Fold increase of Fc ϵ RI positive cells after culture with IgE-rich serum over cells cultured with IgE-free serum. Each bar represents the mean $\pm$ SEM of four independent experiments (* $p < 0.05$).

(8 μ g IgE/ml medium) or heat-inactivated serum. After the culture period, all cells were sensitized for 1 h at 4 $^{\circ}$ C to fully saturate the Fc ϵ RI receptors expressed on the cell surface. The binding of IgE to the cells was detected through a monoclonal anti-IgE antibody. As shown in Fig. 4, the mean percentage of positive cells (cells showing fluorescence signal for Fc ϵ RI expression) was found to be approximately double in the cells that had been incubated with IgE-rich serum compared to those cultured with IgE-free serum (where Fc-IgE chain was denatured by heat), enhancement being statistically significant. All experiments were performed with a control antibody (irrelevant specificity) matching the anti-IgE isotype to demonstrate that there was no unspecific binding of anti-IgE to the cell surface. Finally, the secondary antibody controls showed no fluorescence signal.

4. Discussion

In this study, we evaluated whether the exposure of skin MC to high serum IgE concentration influences two aspects of the canine MC biology: the Fc ϵ RI expression on its surface and their TNF- α production upon an Fc ϵ RI-dependent stimulation. We have shown that mature skin MC cultured for 5 days in a medium supplemented with an increased serum IgE concentration expresses a higher Fc ϵ RI density and release

increased TNF- α concentrations upon immunological stimulation, when compared to cells cultured with IgE-free serum for the same period of time.

We recently succeeded in maintaining isolated mature skin MC viable for 5 days after the addition of canine exogenous SCF to the culture medium (Brazís et al., 2000). This *in vitro* system has allowed us to culture this cell population under different microenvironmental conditions such as increasing serum IgE concentrations, and to observe the subsequent changes in MC activity.

In this study, we have identified, through a flow cytometric technique, the population of dermal cells capable of binding IgE. Although other cell populations such as epidermal Langerhan's cells can bind IgE through the Fc ϵ RI receptors in the human skin (Bieber et al., 1992; Osterhoff et al., 1994), it has been shown that non-lesional skin from healthy patients contains few IgE+ cells, all of them located in the dermis (Klupal et al., 1997). Moreover, immunohistochemical studies have revealed that, in normal canine skin, only MC can be labeled using an anti-canine IgE antibody (Olivry et al., 1996). It therefore seems likely that the IgE-bound positive cells identified in the present study correspond mostly to MC, and the receptor responsible for this binding reflects the Fc ϵ RI receptor (Brazís et al., *in press*).

Fc ϵ RI expression was examined on the surface of canine MC maintained in different conditions (in the presence or absence of IgE). Although the number of MC, counted by toluidine blue staining, was not significantly different between cells maintained under both conditions, the percentage of Fc ϵ RI-positive skin cells was found to be approximately double in cells cultured with IgE serum compared to that cultured in the absence of IgE, after saturation of IgE receptors. This suggested the possibility of differential Fc ϵ RI expression on skin MC, probably due to the presence of canine IgE during the culture period.

One of the most significant consequences of an Fc ϵ RI upregulation is the enhanced-MC mediator release after an IgE-dependent stimulation. This effect was especially relevant when newly formed, rather than preformed, mediators were analyzed (Conroy et al., 1977; Yamaguchi et al., 1997). In order to analyze the functional consequences of maintaining canine MC in an IgE-rich microenvironment, the ability of MC to produce TNF- α was assessed. Skin

MC were cultured for 5 days with medium supplemented with increasing serum IgE concentration; following the culture period, we assessed their ability to release TNF- α upon immunological stimulation. A dose-dependent correlation was observed between the serum IgE concentration added to the medium, and the TNF- α released. These results together with that found by flow cytometry suggested that MC cultured in the presence of high concentrations of IgE were more sensitive to immunological stimulation, probably due to an increase in the IgE molecules bound to the cells. However, in order to further discriminate the specific contribution of IgE to this response, and to rule out the possibility that the enhanced mediator release was due to a general enhancement of the cell responsiveness to any stimulus, new experiments were performed.

When the release of TNF- α by MC cultured in an IgE-rich medium upon an IgE-independent activation was compared to that obtained by cells cultured in an IgE-free medium, no significant differences were observed. However, the parallel activation of the same cells upon anti-IgE showed a marked enhancement in the cytokine production by the cells cultured without IgE. This enhancement was found to be statistically significant.

Taken these results together, there seems to be a positive correlation between the detection of higher number of Fc ϵ RI+ cells and higher concentrations of TNF- α released by MC cultured in the presence of an enhanced serum IgE concentration. These results could suggest that, as found in human and murine cells, the increased Fc ϵ RI density in MC surface may contribute to the binding of increased IgE molecules to the cell surface, leading to an enhanced IgE-mediated production of pro-inflammatory cytokines such as TNF- α .

Finally, this phenomenon could be of particularly importance in finding novel approaches to the treatment of allergic diseases in dogs. Recently, the use of anti-IgE monoclonal antibody therapy has already been shown to be viable for the treatment of canine atopic dermatitis under experimental conditions (Aiyappa et al., 2000).

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